invitrogen™

pcDNA4/TO-E™ Echo™-Adapted Expression Vector

For cloning a gene of interest using the Echo™ Cloning System and inducible expression in mammalian cells using the T-REx™ System.

Catalog nos. ET460-XX

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User Manual

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Kit Contents and Storage

Types of Kits

Several pcDNA4/TO-E™ Echo™ Cloning System Kits are available. The table below lists the kits that include the pcDNA4/TO-E™ Echo™-Adapted Expression Vector.

Kit	Reagents Supplied	Catalog nos.
pcDNA4/TO-E™ Echo™-Adapted Expression Vector Kit	pcDNA4/TO-E™ vector Expression control vector Cre Recombinase and 10X buffer CMV Forward Primer	ET460-01
pcDNA4/TO-E™ Echo™-Adapted Expression Vector Kit with a choice of Donor Vector Kit and One Shot® TOP10 Chemically Competent *E. coli* (see page 25 for more information on donor vectors)	pUni/V5-His TOPO® TA Cloning Kit	ET460-10C
	pUniBlunt/V5-His TOPO® Cloning Kit	ET460-20C
	pUni/V5-His A, B, and C	ET460-30C
	pUniD/V5-His TOPO® Cloning Kit	ET460-40C

Shipping/Storage

The pcDNA4/TO-E™ Echo™-Adapted Vector Kit is shipped on dry ice. Upon receipt, store the pcDNA4/TO-E™ reagents at -20°C. Store the One Shot® Competent *E. coli* at -80°C.

pcDNA4/TO-E™ Reagents

The pcDNA4/TO-E™ reagents are listed below. Store at -20°C.

Item	Concentration	Amount
pcDNA4/TO-E™	Supercoiled, lyophilized in TE, pH 8.0	20 µg
Cre Recombinase	Please check the label on the tube for exact concentration of the enzyme Enzyme supplied in: 50 mM Tris-HCl, pH 8.0 5 mM EDTA 1 mM EGTA 10 mM β-mercaptoethanol 20% Glycerol	12 µl
10X Recombinase Buffer	500 mM Tris-HCl, pH 7.5 100 mM $MgCl_2$ 300 mM NaCl 1.0 mg/ml BSA	25 µl
CMV Forward Primer (21 mer)	Lyophilized in TE Buffer, pH 8.0 (5´-CGCAAATGGGCGGTAGGCGTG-3´)	2 µg (306 pmoles)
Expression Control (pcDNA4/TO-E/Uni-*lacZ*™)	Supercoiled, lyophilized in TE, pH 8.0	20 µg

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One Shot® Reagents (Optional)

The table below describes the items included in the One Shot® Competent *E. coli* kit. **Store at -80°C**.

Item	Concentration	Amount
SOC Medium (may be stored at room temperature or at +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 *E. coli*	--	11 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of TOP10

TOP10: Use this strain for general cloning of your gene of interest. **Note:** This strain cannot be used for growth and transformation of donor vectors.

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Introduction

Overview

Introduction

The Echo™ Cloning System allows direct recombination of your gene of interest downstream of an appropriate promoter for expression in the host system of choice. pcDNA4/TO-E™ is a member of the Echo™ Cloning System family of expression vectors and is specifically designed for inducible expression in mammalian cells. The vector allows tetracycline-regulated expression of the gene of interest in mammalian host cells cotransfected with the pcDNA6/TR vector (Catalog no. V1025-20).

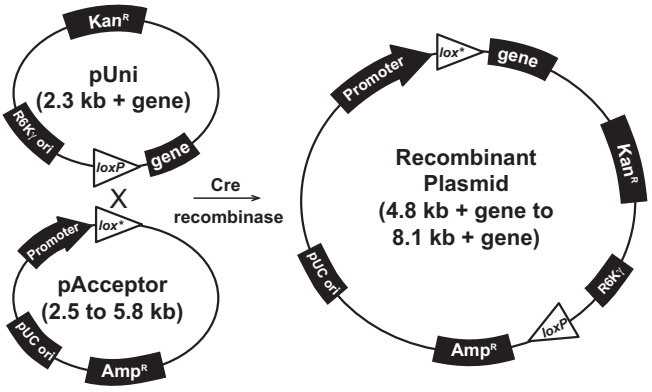
For more information about pcDNA6/TR and the T-REx™ System, please refer to the T-REx™ System manual, our World Wide Web site (www.invitrogen.com), or call Technical Service (see page 29).

Echo™ Cloning System

The Echo™ Cloning System is based on the univector plasmid-fusion system (UPS) described by Elledge and coworkers to quickly and easily recombine a gene of interest into a series of recipient (acceptor) vectors (Liu *et al.*, 1998; Liu *et al.*, 1999). The system consists of the univector (donor) vector containing the gene of interest and recipient (acceptor) vectors containing various regulatory sequences for expression in the host of choice. The Echo™ System utilizes the *cre-lox* site-specific recombination system of bacteriophage P1 (Abremski *et al.*, 1983; Sternberg *et al.*, 1981a). The product of the *cre* gene is a site-specific recombinase that catalyzes conservative recombination between two 34 bp *loxP* or *loxH* sequences to resolve P1 dimers generated by replication of circular lysogens.

Plasmid Fusion

The donor vector and the acceptor vector (i.e. pcDNA4/TO-E™) each contains a *lox* site. The donor vector (pUni) contains a *loxP* site, while the acceptor vector contains either a *loxP* or a *loxH* site (see the next page for more information about *loxH*). You have already constructed the donor vector containing the PCR product of interest via the TOPO® Cloning method. pcDNA4/TO-E™ allows you to regulate expression of your PCR product using the T-REx™ System. The unique *loxH* site is located downstream of the regulatory sequences. By mixing the donor vector containing the PCR product of interest with pcDNA4/TO-E™ in the presence of Cre recombinase, a plasmid fusion is created that expresses the PCR product in mammalian cells. A generic diagram is shown below.



*lox** = *loxP* or *loxH* depending on acceptor vector

Continued on next page

Overview, Continued

loxP or *loxH* Sites

The sequence of the *loxP* site is shown below. The *loxP* site consists of a 34 bp sequence containing two 13 bp inverted repeats (see underlined bases) separated by an 8 bp spacer (Hoess *et al.*, 1982). The inverted repeats may form a stem and loop structure that may reduce expression of the gene of interest in some cases. A variation of the *loxP* site (*loxH*, see below) was created to eliminate the formation of a stem and loop structure and improve expression. Mutated bases are shown in boldface. Please note that some acceptor vectors including pcDNA4/TO-E™ contain a *loxH* site. Cre-mediated recombination can still occur between a *loxP* and a *loxH* site although the efficiency may be slightly reduced.

- *loxP*: ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA T
- *loxH*: AT**T** AC**C** TC**A** TAT AGC ATA CAT TAT ACG AAG TTA T

Cre Recombinase

Cre recombinase (MW = 35 kDa) is a site-specific recombinase that binds to specific sequences (*loxP* and *loxH* sites), brings together the target sites, cleaves, and covalently attaches to the DNA. Recombination occurs following two pairs of strand exchanges and ligation of the DNAs in a novel (recombinant) form. A nucleophilic hydroxylated tyrosine initiates the DNA cleavage event by attack on a specific phosphodiester bond followed by the covalent attachment of the recombinase to the target sequence through a phosphoamino acid bond (Abremski and Hoess, 1992; Argos *et al.*, 1986). The reaction does not require any host factors or ATP, but does require Mg^{2+} or spermidine for activity (Abremski *et al.*, 1983). Recombination between two supercoiled substrates, each containing a *loxP* or *loxH* site, results in a supercoiled dimer. The extent of the reaction is 10-20% and appears to be stoichiometric (Abremski and Hoess, 1984; Abremski *et al.*, 1983).

Selection of Recombinants

By fusing the two plasmids, kanamycin resistance is now linked to the pUC origin of replication. The recombination reaction is transformed into TOP10 *E. coli* and recombinants selected by plating the transformation reaction onto plates containing kanamycin. Because the donor plasmid carries the R6Kγ origin of replication, it will not propagate in *E. coli* such as TOP10 which do not carry the *pir* gene. In addition, the acceptor vector, which carries the ampicillin resistance gene will not be selected. Therefore every colony that is selected on kanamycin will represent a recombined fusion plasmid.

pcDNA4/TO-E™

pcDNA4/TO-E™ is a 5.0 kb vector derived from pcDNA4/TO and designed for high-level, inducible expression in most mammalian hosts. The vector contains the following elements:

- Cytomegalovirus (CMV) immediate-early promoter containing two tetracycline operator 2 ($TetO_2$) sites for tetracycline-regulated expression of your gene of interest in mammalian cells (Yao *et al.*, 1998).
- A *loxH* site for univector plasmid fusion
- Zeocin™ resistance gene for selection of stable cell lines (Mulsant *et al.*, 1988)
- The pUC origin for high-copy replication and maintenance in most *E. coli* strains
- The ampicillin (*bla*) resistance gene for selection in *E. coli*

For a map and a description of pcDNA4/TO-E™, please refer to page 18.

Other Echo™-adapted acceptor vectors are available separately and are provided with their own manuals. For more information on other available acceptor vectors, please visit our Web site (www.invitrogen.com) or call Technical Service (see page 29).

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Overview, Continued

Repression and Derepression

The $TetO_2$ sequences in the pcDNA4/TO-E™ vector serve as binding sites for four Tet repressor molecules (comprising two Tet repressor homodimers) and confer tetracycline-responsiveness to your gene of interest. The Tet repressor is expressed from the pcDNA6/TR plasmid. For more information about the $TetO_2$ sequences, please see below. For more information about the pcDNA6/TR plasmid and the Tet repressor, please refer to the T-REx™ System manual. The T-REx™ System manual is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 29).

In the absence of tetracycline, expression of your gene of interest is repressed by the binding of Tet repressor homodimers to the $TetO_2$ sequences. Addition of tetracycline to the cells derepresses the hybrid CMV/$TetO_2$ promoter in pcDNA4/TO and allows expression of your gene of interest

Tet Operator Sequences

The promoters of bacterial *tet* genes contain two types of operator sequences, O_1 and O_2, that serve as high-affinity binding sites for the Tet repressor (Hillen and Berens, 1994; Hillen *et al.*, 1983). Each O_1 and O_2 site binds to one Tet repressor homodimer. While Tet repressor homodimers bind to both *tet* operators with high affinity, studies have shown that the affinity of the Tet repressor homodimer for O_2 is three- to five-fold higher than it is for O_1 (Hillen and Berens, 1994).

Tet operators have been incorporated into heterologous eukaryotic promoters to allow tetracycline-regulated gene expression in mammalian cells (Gossen and Bujard, 1992; Yao *et al.*, 1998). In the T-REx™ System, two copies of the O_2 operator sequence ($TetO_2$) were inserted into the strong CMV promoter of pcDNA4/TO-E™ to allow regulated expression of your gene of interest by tetracycline. We use the $TetO_2$ operator sequence in pcDNA4/TO-E™ to maximize repression of basal gene expression. For more detailed information about *tet* operators, please refer to Hillen and Berens (1994).

Yao *et al.* (1998) have recently demonstrated that the location of *tet* operator sequences in relation to the TATA box of a heterologous promoter is critical to the function of the *tet* operator. Regulation by tetracycline is only conferred upon a heterologous promoter by proper spacing of the $TetO_2$ sequences from the TATA box (Yao *et al.*, 1998). For this reason, the first nucleotide of the $TetO_2$ operator sequence has been placed 10 nucleotides after the last nucleotide of the TATA element in the CMV promoter in pcDNA4/TO-E™. Please refer to the diagram on page 8 for the sequence and placement of the $TetO_2$ sequences in relation to the TATA box.

In other tetracycline-regulated systems, the $TetO_2$ sequences are located upstream of the TATA element in the promoter of the inducible expression vector (Gossen and Bujard, 1992). These systems differ substantially from the T-REx™ System in that they use regulatory molecules composed of the Tet repressor fused to a viral transactivation domain. The presence of viral transactivation domains appears to overcome the requirement for specific positioning of the $TetO_2$ sequences in relation to the TATA box of the heterologous promoter. However, the presence of viral transactivation domains has been found to have deleterious effects in some mammalian cell lines.

Continued on next page

Experimental Outline

The table below describes the general steps needed to recombine, transform, and express your protein of interest.

Step	Action	Page
1	Perform the recombination reaction using your donor vector and pcDNA4/TO-E™.	5
2	Transform the recombination reaction into competent TOP10 *E. coli* cells.	6
3	Select transformants on LB plates containing 50 µg/ml kanamycin.	7
4	Analyze transformants by restriction digestion.	7
5	Select the correct clone and cotransfect your construct and pcDNA6/TR into the mammalian cell line of interest using your method of choice.	10
6	Induce expression of your recombinant protein with tetracycline and analyze by western blot or functional assay.	10
7	Generate a double stable cell line, if desired.	13
8	Purify your protein, if desired.	15

Methods

Recombining Your Gene into pcDNA4/TO-E™

Introduction

At this point, you should have a plasmid preparation of your donor vector construct in addition to pcDNA4/TO-E™. Please review the information below and on the next page before performing the recombination reaction.

Preparation and Maintenance of pcDNA4/TO-E™

To prepare pcDNA4/TO-E™ for use, add 20 µl sterile water to create a 1 µg/µl stock solution. You can further dilute a small aliquot of plasmid or use the stock solution as is. Store the stock solution at -20°C when you are finished.

If you wish to propagate the pcDNA4/TO-E™ plasmid or prepare plasmid DNA, you may transform the plasmid into TOP10 *E. coli* as described on page 6. Use 10-100 ng of plasmid DNA for transformation and select transformants on LB plates containing 50 to 100 µg/ml ampicillin. Be sure to prepare a glycerol stock of your plasmid-containing TOP10 strain for long-term storage (see page 9).

Before Starting

You will need the following reagents and equipment.

- 100 ng of your donor vector construct
- 100 ng of pcDNA4/TO-E™ (included in kit)
- Microcentrifuge tubes
- Heat blocks set at 37°C and 65°C
- Ice bucket with ice
- Cre recombinase (included in the kit)
- 10X Recombinase Buffer (included in the kit)

Recombination Reaction

1. Set up each 20 µl recombination reaction **on ice** as follows:

Donor vector (100 ng)		x µl
pcDNA4/TO-E™ (100 ng)		y µl
10X Recombinase Buffer		2 µl
Deionized water	add to a total volume of	17 µl
Cre Recombinase		1 µl
Final Volume		20 µl

2. Incubate at 37°C for 20 minutes.
3. Incubate at 65°C for 5 minutes to inactivate the recombinase.
4. Place the tube on ice and proceed to **Transformation**, next page. If you run out of time, you may store the recombination reaction at +4°C or -20°C overnight. Longer storage times have not been tested.

Transforming the Recombination Reaction

Introduction

Once you have performed the recombination reaction, you are ready to transform your *E. coli* host. We recommend using TOP10 *E. coli* (available with the kit) for transformation, but other strains are suitable. *E. coli* strains should be endonuclease A deficient (*end*A) and recombination deficient (*rec*A) to ensure quality plasmid preparations and reduce the chances of recombination, respectively.

Materials Supplied by the User

In addition to general microbiological supplies (i.e. plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath
- LB plates containing 50 µg/ml kanamycin (see **Important**, below)
- 37°C shaking and non-shaking incubator
- SOC (supplied in the One Shot® kit)

Important

It is important to select for the fusion plasmid using kanamycin. Remember that the donor vector contains the R6Kγ origin. This origin can only be maintained in *E. coli* strains containing the *pir* gene. After the donor vector and pcDNA4/TO-E™ acceptor vectors have recombined to form the fusion plasmid, the kanamycin resistance gene (from the donor vector) is linked to the pUC origin (from pcDNA4/TO-E™). The fusion plasmid can be maintained in *E. coli* strains that do not contain the *pir* gene (i.e. TOP10). By selecting for kanamycin resistance, you ensure that only colonies containing the fusion vector are selected.

Preparation for Transformation

The following transformation protocol is for use with the TOP10 One Shot® competent cells available with the kit. If you are using other competent cells, please follow the manufacturer's protocol.

For each transformation, you will need one vial of One Shot® TOP10 competent cells and two selective plates. Perform the following steps before beginning.

- Equilibrate a water bath to 42°C.
- Thaw the vial of SOC medium from the One Shot® box and bring to room temperature.
- Warm LB plates containing 50 µg/ml kanamycin at 37°C for 30 minutes.
- Thaw **on ice** 1 vial of One Shot® cells for each transformation.

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Transforming the Recombination Reaction, Continued

One Shot® Transformation Reaction

1. Add 5 µl of the recombination reaction to a vial of One Shot® TOP10 *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Heat-shock the cells for 30 seconds at 42°C without shaking.
3. Immediately transfer the tubes to ice.
4. Add 500 µl of room temperature SOC medium.
5. Cap the tube tightly and shake the tube horizontally at 37°C for 45 minutes.
6. Spread 50 µl from each transformation onto a prewarmed LB plate containing 50 µg/ml kanamycin. Pellet the remaining cells, resuspend the cell pellet in 50 µl SOC and plate. Incubate overnight at 37°C.
7. An efficient recombination reaction will produce hundreds of colonies. Pick ~5 colonies for analysis.

Analysis of Positive Clones

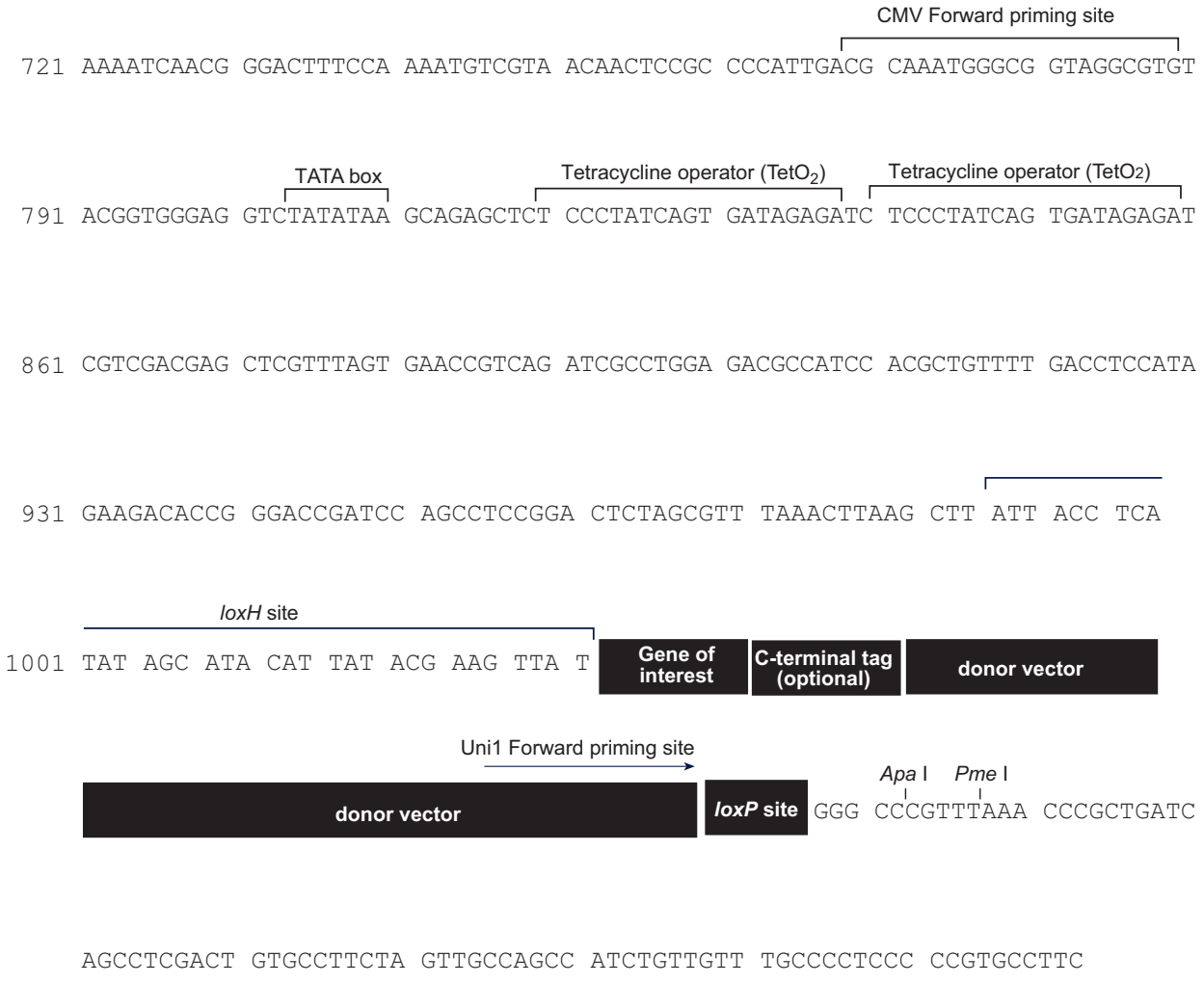
1. Culture the 5 colonies (see previous page) overnight in 2-5 ml LB or SOB medium containing 50 µg/ml kanamycin. See pages 16-17 for recipes for LB and SOB medium.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P. ™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01) or the S.N.A.P. ™ MidiPrep Kit (10-200 µg DNA, Catalog no. K1910-01).
3. Analyze the plasmids by restriction analysis. Use an enzyme or enzymes that cut once in the donor vector and once in the acceptor vector to yield two fragments that are distinguishable from one another. Please note that other strategies are possible.
4. (Optional) To sequence the fusion plasmid to confirm the fusion junctions, use the CMV Forward and Uni1 Forward sequencing primers. Please refer to the diagram on the following page for the sequence around the pcDNA4/TO-E™ *loxH* site. Refer to the donor vector manual for the sequence around the donor vector *loxP* site.

If you need help with setting up restriction enzyme digests or DNA sequencing, please refer to general molecular biology texts (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).

Transforming the Recombination Reaction, Continued

Sequencing Your Construct

The sequence surrounding your insert is shown below. Unique restriction sites are labeled to indicate the cleavage site. Please note that the complete sequence of pcDNA4/TO-E™ is available for downloading from our Web site (www.invitrogen.com) or from Technical Service.


CMV Forward priming site
721 AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG GTAGGCGTGT
TATA box
Tetracycline operator (TetO2)
Tetracycline operator (TetO2)
791 ACGGTGGGAG GTCTATATAA GCAGAGCTCT CCCTATCAGT GATAGAGATC TCCCTATCAG TGATAGAGAT
861 CGTCGACGAG CTCGTTTAGT GAACCGTCAG ATCGCCTGGA GACGCCATCC ACGCTGTTTT GACCTCCATA
931 GAAGACACCG GGACCGATCC AGCCTCCGGA CTCTAGCGTT TAAACTTAAG CTT ATT ACC TCA
loxH site
1001 TAT AGC ATA CAT TAT ACG AAG TTA T
Gene of interest
C-terminal tag (optional)
donor vector
Uni1 Forward priming site
Apa I
Pme I
donor vector
loxP site
GGG CCCGTTTAAA CCCGCTGATC
AGCCTCGACT GTGCCTTCTA GTTGCCAGCC ATCTGTTGTT TGCCCCTCCC CCGTGCCTTC

Continued on next page

Transforming the Recombination Reaction, Continued

Fusion Vector Analysis

It should be clear from restriction analysis that you have a dimer plasmid consisting of the donor vector and pcDNA4/TO-E™. Occasionally, trimers will result. Trimers usually consist of two donor vector molecules and one acceptor molecule. In theory, trimers may result from two sequential fusion events or a single fusion event between a pre-existing monomeric substrate and a dimeric substrate. The production of trimers can be eliminated if gel-purified monomeric supercoiled DNA is used in the recombination reaction. Please note that trimers usually express as well as the dimer product.

Preparing a Glycerol Stock for Long-Term Storage

Once you have identified the correct clone, prepare a glycerol stock for long term storage.

1. Streak out the original colony on LB plates containing 50 µg/ml kanamycin to isolate single colonies.
2. Select a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow overnight until culture is saturated.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C. (You may also want to store a stock of plasmid DNA at -20°C.)

Transfection and Analysis

Introduction

Once you have created the pcDNA4/TO-E™ fusion plasmid, have verified its integrity, and have prepared clean plasmid preparations of both the fusion plasmid and pcDNA6/TR, you are ready to cotransfect the plasmids into the mammalian cell line of choice. Please refer to the T-REx™ System manual for complete information on pcDNA6/TR, transfection, and induction of expression. We recommend that you include the positive expression control vector and a mock transfection (negative control) to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipids decreasing the transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P. ™ MidiPrep Kit (Catalog no. K1910-01) or other resin-based method.

Important

Because tetracycline-regulated expression in the T-REx™ System is based on a repression/derepression mechanism, the amount of Tet repressor that is expressed in the host cell line from pcDNA6/TR will determine the level of transcriptional repression of the Tet operator sequences in your pcDNA4/TO-E™ fusion vector. Tet repressor levels should be sufficiently high to suitably repress basal level transcription. We recommend that you cotransfect your mammalian host cell line with a ratio of **at least 6:1** (w/w) pcDNA6/TR:pcDNA4/TO-E™ fusion vector DNA. You may want to try varying ratios of pcDNA6/TR:pcDNA4/TO-E™ fusion vector to optimize repression and expression for your particular cell line and your gene of interest.

Cotransfection and Induction with Tetracycline

General guidelines are provided below to cotransfect your pcDNA4/TO-E™ fusion vector (or the control plasmid) and pcDNA6/TR into your cell line of interest and to induce expression of your protein of interest with tetracycline. Please refer to the T-REx™ System manual for more information on transfection and the preparation and handling of tetracycline.

- Use cells that are approximately 60% confluent for transfection.
- Cotransfect your pcDNA4/TO-E™ fusion vector and pcDNA6/TR at a ratio of 6:1 (w:w) into the cell line of choice using your preferred method. Absolute amounts of plasmid used for transfection will vary depending on the method of transfection and the cell line used.
- After transfection, add fresh medium and allow the cells to recover for 24 hours before induction.
- Remove medium and add fresh medium containing the appropriate concentration of tetracycline to the cells. In general, we recommend that you add tetracycline to a final concentration of 1 µg/ml (5 µl of a 1 mg/ml stock solution per 5 ml of medium) to the cells and incubate the cells for 24 hours at 37°C.
- Harvest the cells and assay for expression of your gene of interest.

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Positive Expression Control

pcDNA4/TO-E/Uni-*lacZ*™ is provided as a positive control vector for mammalian cell transfection and expression and may be used to optimize transfection conditions for your cell line. Cotransfection of the positive control vector and pcDNA6/TR results in the expression of β-galactosidase following the addition of tetracycline. A successful cotransfection will result in positive β-galactosidase expression and can be easily assayed by staining with X-gal (see below).

Assay for β-galactosidase Activity

You may assay for β-galactosidase expression by activity assay using cell-free lysates (Miller, 1972) or by staining the cells for activity. Invitrogen offers the β-Gal Assay Kit (Catalog no. K1455-01) and the β-Gal Staining Kit (Catalog no. K1465-01) for fast and easy detection of β-galactosidase expression.

Detection of β-Galactosidase

If you wish to detect expression of β-galactosidase by western blot, you may use an antibody to β-galactosidase.

Detection of Recombinant Fusion Proteins

If you have expressed your protein as a fusion to the C-terminal V5 epitope and polyhistidine (6xHis) tag, you can detect expression using the Anti-V5 or Anti-His(C-term) antibodies (see page 25 for ordering information). You may also use an antibody to your protein of interest.

To detect your fusion protein by western blot, you will need to prepare a cell lysate from transfected cells. We recommend that you perform a time course to optimize expression of your fusion protein (e.g. 12, 24, 48, 72 hours, etc. after transfection). Use the protocol below to lyse cells. Other protocols and lysis buffers are also suitable.

1. Wash cell monolayers (~5 x 10^5 to 1 x 10^6 cells) once with phosphate-buffered saline (PBS, see recipe on the next page).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend pellet in 50 µl Cell Lysis Buffer (see recipe on the next page) and vortex.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells. **Note:** You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
5. Centrifuge the cell lysate at 10,000 x g for 10 minutes to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration. **Note:** Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Add SDS-PAGE sample buffer to a final concentration of 1X and boil the sample for 5 minutes.
7. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.

Continued on next page

Note

The C-terminal peptide containing the V5 epitope and the polyhistidine (6xHis) tag will add approximately 5 kDa to the size of your protein.

Cell Lysis Buffer

50 mM Tris, pH 7.8

150 mM NaCl

1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions. For 100 ml, combine:

1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml

2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.

Note: Just before use, add protease inhibitors to a small amount of buffer at the following final concentrations:

1 mM PMSF

1 µg/ml pepstatin

1 µg/ml leupeptin

Phosphate-Buffered Saline (PBS)

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

1.8 mM KH_2PO_4

1. Dissolve the following in 800 ml of deionized water:

   8 g NaCl

   0.2 g KCl

   1.44 g Na_2HPO_4

   0.24 g KH_2PO_4
2. Adjust pH to 7.4 with concentrated HCl.
3. Bring the volume up to 1 liter and autoclave for 20 minutes on liquid cycle.
4. Store at +4°C or room temperature.

Creation of Stable Cell Lines

Introduction

Once you have established that your construct can be inducibly expressed, you may create a stable cell line that carries the tet repressor and inducibly expresses your gene of interest. pcDNA4/TO-E™ contains the Zeocin™ resistance gene to allow selection of stable lines using Zeocin™, pcDNA6/TO carries the blasticidin resistance gene. Before establishing a double-stable cell line, it is important to determine the minimum concentration of selection agents required to kill untransfected cells. The protocol below provides information for determining the appropriate concentration of Zeocin™. A similar protocol for blasticidin can be found in the T-REx™ System manual.

Note

Please note that your gene of interest will be constitutively expressed if you transfect your pcDNA4/TO-E™ fusion vector into mammalian host cells prior to transfecting the pcDNA6/TR plasmid. For more information on selection of stable cell lines using pcDNA6/TR and blasticidin, please refer to the T-REx™ System manual.

Reminder: When generating a stable cell line expressing the Tet repressor (from pcDNA6/TR), you will want to select for clones that express the highest levels of Tet repressor to use as hosts for your pcDNA4/TO-E™ fusion vector. Those clones that express the highest levels of Tet repressor should exhibit the most complete repression of basal transcription of your gene of interest.

Determination of Antibiotic Sensitivity

To generate a stable cell line expressing your protein of interest, you need to determine the minimum concentration of Zeocin™ required to kill your untransfected host cell line. Typically, concentrations between 50 and 1000 μg/ml Zeocin™ are sufficient to kill the untransfected host cell line. Test a range of concentrations (see below) to ensure that you determine the minimum concentration necessary for your cell line. For more information on Zeocin™ including instructions on preparation and storage, please refer to page 21.

Note: Before transfecting your host cell line with pcDNA6/TR, you will need to perform a similar experiment to determine the minimum concentration of blasticidin required to kill the untransfected cell line. Please refer to the T-REx™ System manual for information about blasticidin.

- Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates.
- The next day, substitute culture medium with medium containing varying concentrations of Zeocin™ (e.g. 0, 50, 125, 250, 500, 750, and 1000 μg/ml).
- Replenish the selective medium every 3-4 days, and observe the percentage of surviving cells.
- Count the number of viable cells at regular intervals to determine the appropriate concentration of Zeocin™ that prevents growth within 1-2 weeks after addition of Zeocin™.

Creation of Stable Cell Lines, Continued

Effect of Zeocin™ on Sensitive and Resistant Cells

The method of killing of Zeocin™ is quite different from blasticidin, neomycin, and hygromycin**. Cells do not round up and detach from the plate.** Sensitive cells may exhibit the following morphological changes upon exposure to Zeocin™:

- Vast increase in size (similar to the effects of cytomegalovirus infecting permissive cells)
- Abnormal cell shape
- Presence of large empty vesicles in the cytoplasm (breakdown of the endoplasmic reticulum and golgi apparatus or scaffolding proteins)
- Breakdown of plasma and nuclear membrane (appearance of many holes in these membranes)

Eventually, these "cells" will completely break down and only "strings" of protein will remain.

Zeocin™-resistant cells should continue to divide at regular intervals to form distinct colonies. There should not be any distinct morphological changes in Zeocin™-resistant cells when compared to cells not under selection with Zeocin™. For more information about Zeocin™, please see page 21.

Plasmid Linearization

We have found that it is not necessary to linearize the pcDNA4/TO-E™ fusion vector prior to transfection.

Selection of Stable Integrants

Once you have determined the appropriate Zeocin™ concentration to use for selection, you can generate a stable cell line expressing pcDNA6/TR and your pcDNA4/TO-E™ fusion vector. We recommend that you first generate a stable cell line expressing pcDNA6/TR and then use this cell line as the host for your pcDNA4/TO-E™ fusion vector.

1. Once you have obtained a stable cell line expressing the Tet repressor, follow the steps below to transfect your stable cell line with the pcDNA4/TO-E™ fusion vector. Use Zeocin™ to select for double stable clones. Remember to maintain your cells in medium containing blasticidin as well.
2. Transfect your cell line of choice with your pcDNA4/TO-E™ fusion vector using the desired protocol. Include a sample of untransfected cells as a negative control.
3. 24 hours after transfection, wash the cells and add fresh medium to the cells.
4. 48 hours after transfection, split the cells into fresh medium containing Zeocin™ at the appropriate concentration for your cell line. Split the cells such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the untransfected cells.
5. Replenish selective medium every 3-4 days until Zeocin™-resistant colonies are detected.
6. Pick at least 20 foci and expand them to test for tetracycline-inducible gene expression.

Continued on next page

Creation of Stable Cell Lines, Continued

Dual Selection of Stable Integrants

If you wish to select for stable cell lines by dual selection, you may cotransfect your pcDNA4/TO-E™ fusion vector and pcDNA6/TR into your cell line of choice and select with Zeocin™ and blasticidin. Pick and expand at least 40 foci to screen for tetracycline-regulated expression of your gene of interest.

Purification

If you have expressed your protein as a fusion to the C-terminal polyhistidine (6xHis) tag, you can purify it using ProBond™ Resin (or other metal-chelating resin). Please refer to the manufacturer's instructions before attempting to purify your fusion protein.

Preparation of Cells for Lysis

Use the procedure below to prepare cells for lysis if you will be purifying your protein on ProBond™ Resin. You will need 5×10^6 to 1×10^7 stably transfected cells for purification of your protein on a 2 ml ProBond™ column (see ProBond™ Protein Purification manual).

1. Seed cells in either five T-75 flasks or 2 to 3 T-175 flasks.
2. Grow the cells in selective medium until they are approximately 80% confluent.
3. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
4. Inactivate the trypsin by diluting with fresh medium (if necessary) and transfer the cells to a microcentrifuge tube.
5. Centrifuge the cells at 1500 rpm for 5 minutes. Resuspend the cell pellet in PBS.
6. Centrifuge the cells at 1500 rpm for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at -70°C until needed.

Lysis of Cells

If you are using ProBond™ resin, refer to the ProBond™ Protein Purification manual for details about sample preparation for chromatography.

If you are using other metal-chelating resin, please refer to the manufacturer's instructions for recommendations on sample preparation.

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone

0.5% Yeast Extract

1.0% NaCl

pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50 µg/ml of kanamycin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

Low Salt LB Medium with Zeocin™

For Zeocin™ to be active, the salt concentration of the medium must be low (< 90 mM) and the pH must be 7.5. Use the medium below to prepare plates and liquid medium for selection in *E. coli*. **Failure to use low salt LB medium will result in non-selection due to inactivation of the drug.**

10 g Tryptone

5 g NaCl

5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 ml. Adjust pH to 7.5 with 5 M NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. Thaw Zeocin™ on ice and vortex before removing an aliquot.
4. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 25-50 µg/ml final concentration.
5. Store plates at +4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.

Continued on next page

SOB Medium (with Kanamycin)

SOB (per liter)

2% Tryptone

0.5% Yeast Extract

0.05% NaCl

2.5 mM KCl

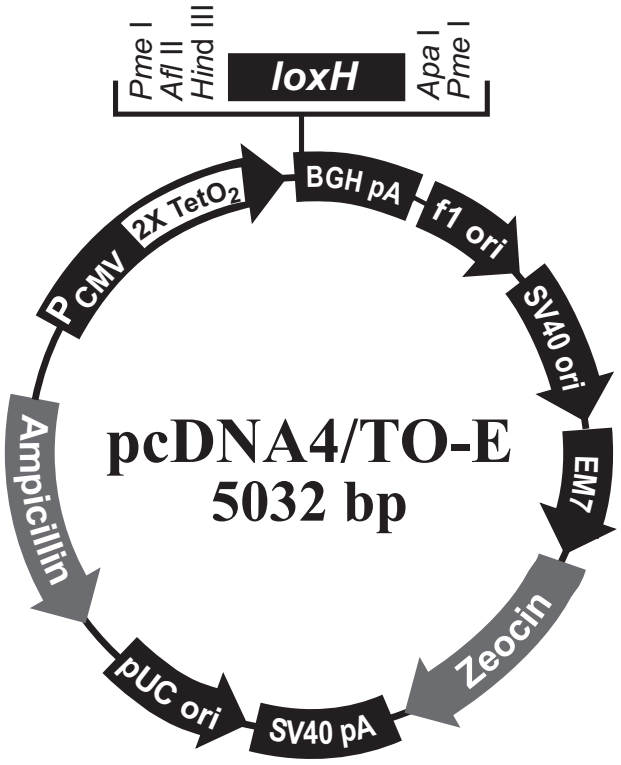
10 mM $MgCl_2$

1. Dissolve 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 950 ml deionized water.
2. Make a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 ml of deionized water. Add 10 ml of this stock KCl solution to the solution in Step 1.
3. Adjust pH to 7.5 with 5 M NaOH and add deionized water to 1 liter.
4. Autoclave this solution, cool to ~55°C, and add 10 ml of sterile 1 M $MgCl_2$. You may also add kanamycin to 50 µg/ml.
5. Store at +4°C. Medium is stable for only ~1 month.

pcDNA4/TO-E™ Vector

Map of pcDNA4/TO-E™

The figure below summarizes the features of the pcDNA4/TO-E™ vector. **The complete sequence for pcDNA4/TO-E™ is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 29)** Details of the sequences surrounding the *loxH* site in pcDNA4/TO-E™ may be found on page 8.



Comments for pcDNA4/TO-E:
5032 nucleotides

CMV promoter: bases 232-958
TATA box: bases 804-810
Tetracycline operator 2 (2X $TetO_2$) sequences: bases 820-859
loxH site: bases 984-1017
BGH polyadenylation sequence: bases 1049-1273
f1 origin: bases 1319-1747
SV40 early promoter and origin: bases 1752-2095
EM7 promoter: bases 2137-2203
Zeocin™ resistance gene: bases 2204-2578
SV40 early polyadenylation sequence: bases 2708-2838
pUC origin: bases 3221-3891 (complementary strand)
bla promoter: bases 4897-4995 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4036-4896 (complementary strand)

Continued on next page

pcDNA4/TO-E™ Vector, Continued

Features of pcDNA4/TO-E™

pcDNA4/TO-E™ (5032 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate early promoter	Permits high-level expression of your gene of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
CMV Forward priming site	Allows sequencing in the sense orientation
Tetracycline operator (O_2) sequences	Two tandem 19 nucleotide repeats which serve as binding sites for Tet repressor homodimers (Hillen and Berens, 1994)
loxH site	Allows recombination between the donor vector and pcDNA4/TO-E™ (Hoess *et al.*, 1982)
BGH reverse priming site	Permits sequencing through the insert
Bovine growth hormone (BGH) polyadenylation sequence	Permits efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992).
f1 origin	Allows rescue of single-strand DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing SV40 large T antigen
EM-7 promoter	Synthetic prokaryotic promoter for expression of the Zeocin™ resistance gene in *E. coli*
Zeocin™ resistance (*Sh ble*) gene (expressed from the SV40 early promoter or the EM-7 promoter)	Permits selection of stable transfectants in mammalian cells and transformants in *E. coli* (Drocourt *et al.*, 1990; Mulsant *et al.*, 1988)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Permits high-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene
Ampicillin resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

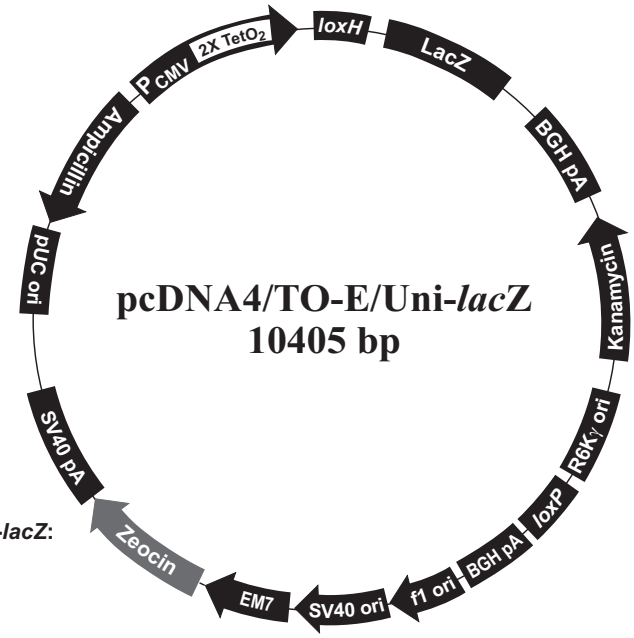
pcDNA4/TO-E/Uni-*lacZ*™

Description

pcDNA4/TO-E/Uni-*lacZ*™ is a 10405 bp expression control vector that contains the *lacZ* gene (3056 bp). The *lacZ* gene was amplified and TOPO® Cloned into pUni/V5-His/Gene-TOPO®. The resulting vector was recombined with pcDNA4/TO-E™ using *cre* recombinase to create pcDNA4/TO-E/Uni-*lacZ*™. **Note**: pUni/V5-His/Gene-TOPO® is similar to pUni/V5-His-TOPO® except that it contains additional DNA between the TOPO® Cloning site and the V5 epitope.

Map of Expression Control Vector

The figure below summarizes the features of the pcDNA4/TO-E/Uni-*lacZ*™ vector. **The complete nucleotide sequence for pcDNA4/TO-E/Uni-*lacZ*™ is available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service (see page 29).**



Comments for pcDNA4/TO-E/Uni-*lacZ*: 10405 nucleotides

CMV promoter: bases 232-958
TATA box: bases 804-810
Tetracycline operator 2 (2X $TetO_2$) sequences: bases 820-859
loxH site: bases 984-1017
LacZ ORF: bases 1041-4097
BGH polyadenylation sequence: bases 4260-4468
Kanamycin promoter: bases 5585-5722 (complementary strand)
Kanamycin resistance gene: bases 4790-5584 (complementary strand)
R6Kγ origin: bases 5940-6342
loxP site: bases 6357-6390
BGH polyadenylation sequence: bases 6422-6646
f1 origin: bases 6692-7120
SV40 early promoter and origin: bases 7125-7468
EM7 promoter: bases 7510-7576
Zeocin™ resistance gene: bases 7577-7951
SV40 early polyadenylation sequence: bases 8081-8211
pUC origin: bases 8594-9264 (complementary strand)
bla promoter: bases 10270-10368 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 9409-10269 (complementary strand)

Zeocin™

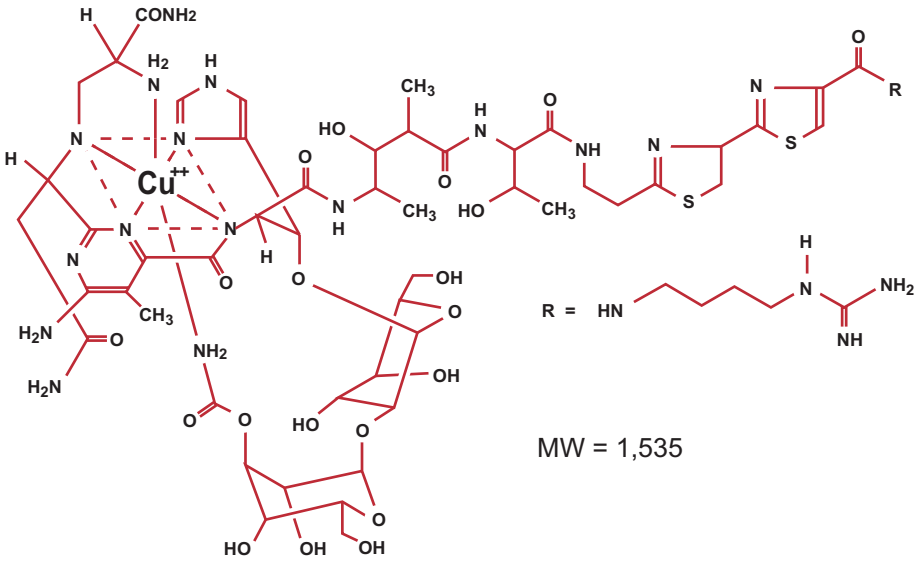
Zeocin™

Zeocin™ belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong antibacterial and antitumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells (Baron *et al.*, 1992; Drocourt *et al.*, 1990; Mulsant *et al.*, 1988; Perez *et al.*, 1989).

The Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13.7 kDa protein that binds Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{60}H_{89}N_{21}O_{21}S_3$ and the molecular weight is 1,535. The diagram below shows the structure of Zeocin™.



Applications of Zeocin™

Zeocin™ is used for selection in mammalian cells (Mulsant *et al.*, 1988), plants (Perez *et al.*, 1989), yeast (Baron *et al.*, 1992), and prokaryotes (Drocourt *et al.*, 1990). Suggested concentrations of Zeocin™ for selection in mammalian cell lines and *E. coli* are listed below:

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25-50 μg/ml in **low salt LB** medium*
Mammalian Cells	50-1000 μg/ml (varies with cell line)

*Efficient selection requires that the concentration of NaCl be no more than 5 g/liter (< 90 mM).

Continued on next page

Handling Zeocin™

- **High salt and acidity or basicity inactivate Zeocin™.** Therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see **Low Salt LB Medium,** page 16). Please note that the pH and salt concentration do not need to be adjusted when preparing tissue culture medium containing Zeocin™.
- Store Zeocin™ at -20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store the drug, and plates or medium containing drug, in the dark at +4°C. Culture medium containing Zeocin™ may be stored at +4°C protected from exposure to light for up to 1 month.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling Zeocin™-containing solutions.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

Preparing and Storing Zeocin™

Zeocin™ is available from Invitrogen (see page 24 for ordering information). For your convenience, Zeocin™ is prepared in autoclaved, deionized water in 1.25 ml aliquots at a concentration of 100 mg/ml. The stability of Zeocin™ is guaranteed for six months, if stored at -20°C protected from exposure to light.

Product Qualification

Vectors

pcDNA4/TO-E™ and pcDNA4/TO-E/Uni-*lacZ*™ are qualified by restriction digest. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pcDNA4/TO-E™	pcDNA4/TO-E/Uni-*lacZ*™
Avr II (linearizes)	5032 bp	Not tested
Bgl II	4208 bp, 824 bp	Not tested
*Hin*d III	5032 bp	5327 bp, 5078 bp
*Bam*H I (linearizes)	Not tested	10405 bp
Pme I	Not tested	5429 bp, 4976 bp

Primers

The CMV Forward Sequencing Primer has been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

Cre Recombinase

Purity: >95% homogeneity

Endonuclease activity: Negative

Exonuclease activity: Negative

Functional Assay: Cre recombinase is qualified using the assay on page 23 of this manual. The donor vector is pUni/*lacZ* and the acceptor vector is pcDNA3.1-E. Five microliters of the recombination reaction is transformed into 50 µl TOP10 One Shot® competent *E. coli* using the protocol on page 7. Twenty-five µl of the transformation reaction is plated on LB plates containing 50 µg/ml kanamycin (performed in duplicate). One microliter of Cre recombinase should yield >500 blue, kanamycin-resistant transformants.

One Shot® Competent *E. coli*

All competent cells are qualified as follows:

- Cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA for chemically competent cells and >1 x 10^9 for electrocompetent cells.
- To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
- Untransformed cells are plated on LB plates 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

Related Products

Additional Products

The T-REx™ Echo™ Expression Support Kit and individual reagents for inducible expression of your gene of interest from the pcDNA4/TO-E™ fusion vector are available from Invitrogen. Ordering information is provided below.

T-REx™ Echo™ Expression System Core Kit

The T-REx™ Echo™ Expression System Support Kit contains the pcDNA6/TR vector which expresses the Tet repressor, Zeocin™, blasticidin, tetracycline, and a comprehensive instruction manual detailing the procedure for inducible expression in mammalian cell lines.

Item	Catalog no.
T-REx™ Echo™ Expression System Support Kit	K1020-03

T-REx™ Cell Lines

For your convenience, Invitrogen offers four mammalian cell lines that stably express the Tet repressor. Expression of your gene of interest from pcDNA4/TO may be assayed by transfection of your pcDNA4/TO construct into any of the T-REx™ cell lines and induction with tetracycline. Ordering information is provided below.

Cell Line	Source	Catalog no.
T-REx™-293	Human embryonic kidney	R710-07
T-REx™-HeLa	Human cervical adenocarcinoma	R714-07
T-REx™-CHO	Chinese hamster ovary cells	R718-07
T-REx™-Jurkat	Human lymphocyte	R722-07

T-REx™ System Components

Many of the reagents used in the T-REx™ System are available separately from Invitrogen. See the table below for ordering information.

Item	Amount	Catalog no.
pcDNA6/TR	20 µg, lyophilized	V1025-20
Tetracycline	5 g, powder	Q100-19
Zeocin™	1 g	R250-01
	5 g	R250-05
Blasticidin	50 mg, powder	R210-01

Continued on next page

Related Products, Continued

Additional Products

Many of the reagents supplied with the pcDNA4/TO-E™ Echo™-Adapted Expression Vector, as well as additional reagents that may be used with the pcDNA4/TO-E™, are available separately from Invitrogen. Ordering information is provided below.

Product	Amount	Catalog no.
PIR1 One Shot® *E. coli* (chemically competent)	11 x 50 µl	C1010-10
Cre Recombinase	10 reactions	R100-10
Anti-His(C-term) Antibody	50 µl*	R930-25
Anti-His(C-term)-HRP Antibody	50 µl*	R931-25
Anti-V5 Antibody	50 µl*	R960-25
Anti-V5-HRP Antibody	50 µl*	R961-25
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K853-01
ProBond™ Purification System with Anti-V5-HRP Antibody	1 kit	K854-01
ProBond™ Metal-Binding Resin	50 ml 150 ml	R801-01 R801-15
Purification Columns	50	R640-50
One Shot® TOP10 chemically competent *E. coli*	21 x 50 µl	C4040-03

*Quantity supplied is sufficient for 25 western blots.

Donor Vectors

The table below lists a variety of donor vectors currently available from Invitrogen to facilitate cloning of your gene of interest for use with Echo™ Cloning System.

Product	Application	Quantity	Catalog no.
pUni/V5-His-TOPO® TA Cloning Kit	Cloning A-tailed PCR products	10 reactions	ET001-10
pUniBlunt/V5-His-TOPO® Cloning Kit	Cloning blunt PCR products	10 reactions	ET002-10
pUniD/V5-His-TOPO® Cloning Kit	Directional cloning of blunt PCR products	10 reactions	ET004-10
pUni/V5-His A, B, and C	Cloning DNA fragments using restriction enzymes	10 reactions	ET003-10

Purchaser Notification

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Limited Use Label License No: 22 Vectors and Clones Encoding Histidine Hexamer

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Continued on next page

Purchaser Notification, Continued

Limited Use Label License No: 119 Echo™ Cloning Products	No license is conveyed to use this product with any recombination sites other than those purchased from Life Technologies Corporation or its authorized distributor. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose.

Technical Service

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

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Material Data Safety Sheets (MSDSs)

MSDSs are available on our Web site at www.invitrogen.com. On the home page, click on **Technical Resources** and follow instructions on the page to download the MSDS for your product.

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